Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 150: 620–631. 2014 ISSN 0031-9317

AtFKBP16-1, a chloroplast lumenal immunophilin, mediates response to photosynthetic stress by regulating PsaL stability

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Received 10 August 2013;
revised 24 September 2013

doi:10.1111/ppl.12116

Arabidopsis contains 16 putative chloroplast lumen-targeted immunophilins (IMMs). Proteomic analysis has enabled the subcellular localization of IMMs experimentally, but the exact biological and physiological roles of most luminal IMMs remain to be discovered. FK506-binding protein (FKBP) 16-1, one of the lumenal IMMs containing poorly conserved amino acid residues for peptidyl-prolyl isomerase (PPIase) activity, was shown to play a possible role in chloroplast biogenesis in Arabidopsis, and was also found to interact with PsaL in wheat. In this study, further evidence is provided for the notion that Arabidopsis FKBP16-1 (AtFKBP16-1) is transcriptionally and post-transcriptionally regulated by environmental stresses including high light (HL) intensity, and that overexpression of AtFKBP16-1 plants exhibited increased photosynthetic stress tolerance. A blue native-polyacrylamide gel electrophoresis/two-dimensional (BN-PAGE/2-D) analysis revealed that the increase of AtFKBP16-1 affected the levels of photosystem I (PSI)-light harvesting complex I (LHCI) and PSI-LHCI-light harvesting complex II (LHCII) supercomplex, and consequently enhanced tolerance under conditions of HL stress. In addition, plants overexpressing AtFKBP16-1 showed increased accumulation of PsaL protein and enhanced drought tolerance. Using a protease protection assay, AtFKBP16-1 protein was found to have a role in PsaL stability. The AtPsaL levels also responded to abiotic stresses derived from drought, and from methyl viologen stresses in wild-type plants. Taken together, these results suggest that AtFKBP16-1 plays a role in the acclimation of plants under photosynthetic stress conditions, probably by regulating PsaL stability.

Abbreviations – ABA, abscisic acid; AtFKBP16-1, Arabidopsis FKBP16-1; BN-PAGE/2-D, blue native-polyacrylamide gel electrophoresis/two-dimensional; Col-0, Columbia-0; CYP, cyclosporine A-binding protein; DEX, dexamethasone; EDTA, ethylenediaminetetraacetic acid; FKBP, FK506-binding protein; Fv/Fm, PSII efficiency; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; HA, hemagglutinin; HL, high light; IMMs, immunophilins; LHCI, light harvesting complex I; LHCII, light harvesting complex II; MOPS-KOH, 3-(N-morpholino)pro-panesulfonic acid-potassium hydroxide; MS, Murashige and Skoog; MV, methyl viologen; NL, normal light; OE, overexpression; PC, plastocyanin; PPIase, peptidyl-prolyl *cis-trans* isomerase; PSI, photosystem I; PSII, photosystem II; PVDF, polyvinylidene difluoride; RNAi, inverted-repeat RNA; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; VC, vector control.

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Introduction

The cellular compartment known as the chloroplast lumen plays a central role in oxygen evolution and the formation of the thylakoid proton gradient, which drives ATP synthesis during oxygenic photosynthesis. The chloroplast lumen is also considered to be vital for balancing the ion currents established by the thylakoid membranes (Pottosin and Schönknecht 1995, Pottosin and Schönknecht 1996). For many years, before proteomic studies were performed, very few lumenal proteins were identified, apart from those proteins involved in photosynthetic reactions. Among the proteins involved in photosynthesis are the oxygen-evolving complex proteins (PsbO, PsbP and PsbQ), which are extrinsic photosystem II (PSII) proteins, as well as plastocyanin (PC), violaxanthin de-epoxidase (Hager and Holocher 1994) and polyphenol oxidase (Sommer et al. 1994, Sokolenko et al. 1995), an extrinsic photosystem I (PSI) PsaN protein (He and Malkin 1992). The chloroplast lumen is also the site of the carboxyl-terminal processing of the PSII D1 protein (Oelmüller et al. 1996). Nevertheless, the chloroplast lumen was not thought to house a network of biochemical events, as is the case for the stroma. Two recent proteomic studies of chloroplast lumen have addressed the possibility that the chloroplast lumen might be important for the regulation of photosynthesis (Schubert et al. 2002, Peltier et al. 2002).

Proteome analysis of the chloroplast lumen of Arabidopsis and spinach revealed many novel lumenal proteins. These are members of the immunophilins [IMMs; seven FK506-binding protein (FKBP)-type and three cyclosporine-binding protein (CYP)-type proteins], DegQ proteases, PsbP domains, pentapeptide proteins and plant ascorbate peroxidase, as well as proteins with unknown function (Kieselbach and Schröder 2003). IMMs, the largest family of proteins in the chloroplast lumen, comprise a ubiquitous protein family containing a peptidyl-prolyl *cis-trans* isomerase (PPIase) domain that facilitates *cis-trans* isomerization of proline imidic peptide bonds, which is a rate-limiting step during protein folding and a critical determinant of protein structure (Lang et al. 1987). Arabidopsis contains 18 putative chloroplast-targeted IMMs, including two in the stroma and 16 in the thylakoid lumen (He et al. 2004). Previous proteomic analysis has revealed that up to 15 genes are localized to the chloroplast (Lippuner et al. 1994, Peltier et al. 2002, Schubert et al. 2002, Friso et al. 2004). The rice genome also contains chloroplast-targeted genes, including 11 FKBPs and 7 CYPs (Ahn et al. 2010). These thylakoid lumen IMMs have specific functions, including the assembly and maintenance of photosynthetic protein/complex during photosynthesis. Two thylakoid lumen IMMs, CYP38 and FKBP20-2, play roles in PSII biogenesis, assembly and maintenance (Lima et al. 2006, Fu et al. 2007, Sirpiö et al. 2008). AtCYP20-2 and OsCYP20-2 are light-regulated and are associated with both PSII and the NAD(H) dehydrogenase complex in the thylakoid lumen (Romano et al. 2004a, 2004b, 2005, Sirpiö et al. 2009, Kim et al. 2012). AtFKBP13 functions in the accumulation of Rieske protein, a subunit of the cytochrome b_6f complex and a redox protein regulated by light intensity in the lumen (Gupta et al. 2002, Gopalan et al. 2004). Recently, AtFKBP16-2 was found to belong to the NAD(H) dehydrogenase complex and was shown to play a key role in the stability of this complex (Peng et al. 2009). OsFKBP16-3 plays a role in environmental stress tolerance in Arabidopsis and rice (Park et al. 2013).

Most lumen IMMs lack the essential amino acid residues required for PPIase enzyme activity. Intriguingly, two major IMMs in the lumen with PPIase activity, FKBP13 and CYP20-2, do not play important roles in photosynthesis. Single and double knockout mutants for these genes did not exhibit altered phenotypes (Ingelsson et al. 2009). This result led to the proposal that PPIase activity is not an essential function of IMMs, and that they have other functions such as those of chaperonins or of enzymes other than PPIase. Arabidopsis FKBP16-1 (AtFKBP16-1) is a putative chloroplast lumenal protein and is unlikely to function as a PPIase because the amino acids essential for PPIase activity are not well-conserved in this protein as they are in most FKBP-type IMMs (Kieselbach and Schröder 2003, He et al. 2004, Gollan et al. 2011). Recently, it was suggested that this protein functions in chloroplast biogenesis, as determined from in silico analysis and that it enables interaction of PsaL with FKBP16-1, as determined by yeast two-hybrid analysis in wheat (Gollan and Bhave 2010, Gollan et al. 2011). However, the exact function of FKBP16-1 is still not known, particularly with respect to PsaL.

In the meantime, in cyanobacteria, PsaL is involved in the trimerization of the PSI complex (Chitnis and Chitnis 1993). However, a trimer of the PSI complex has not been found in higher plants, and it was suggested that PsaL plays a role in the stabilization of PsaH and PsaO (Zhang and Scheller 2004). PsaL (along with Psa-H, I and O) is a peripheral protein on the left side of the PSI complex, but the inhibition of the genes encoding these proteins did not result in any change in photoautotrophic growth, and such plants were fully fertile (Jensen et al. 2003). Arabidopsis plants lacking PsaH were unable to perform state transitions: i.e. light-dependent transitions in which light harvesting complex II (LHCII) dissociates from PSII-LHCII and moves to the PSI complex to form PSI-LHCI-LHCII. The state transitions of plants lacking

PsaL were also affected, although to a lesser extent in these plants than in PsaH knockout plants (Lunde et al. 2000). Thus, PsaH and PsaL are believed to function as docking sites for LHCII (Lunde et al. 2000, Zhang and Scheller 2004, Qin et al. 2011).

In this study, we verified that AtFKBP16-1, a lumenal protein, is regulated transcriptionally and post-transcriptionally by abiotic stresses such as high light (HL), methyl viologen (MV) and hydrogen peroxide (H_2O_2). The overexpression (OE) of AtFKBP16-1 in transgenic plants produced enhanced tolerance to these abiotic stresses. Using BN-PAGE/2-D analysis, we confirmed that the enhancement of AtFKBP16-1 affected the levels of PSI-LHCI and PSI-LHCI-LHCII supercomplex proteins under HL conditions. In addition, FKBP16-1 OE plants showed drought tolerance and increased expression of PsaL protein. Probably this protein was protected from protease degradation under stress conditions in these plants. From a protease protection analysis, it was determined that the in vivo or in vitro AtFKBP16-1 protein functions in maintenance of chloroplast PsaL stability. The results concur with the conclusion that AtFKBP16-1 is a luminal IMM that plays a role in the acclimation of plants under photosynthetic stress conditions, probably by affecting PSI-LHCI-LHCII supercomplex formation via the regulation of PsaL stability.

Materials and methods

Isolation of thylakoid lumenal subfraction

Isolated chloroplasts were fractionated into thylakoid lumen and membrane sub-compartments as described by Kieselbach et al. (1998) with minor modifications. To isolate chloroplasts, Arabidopsis leaves were ground for 30 s in 330 m*M* sorbitol, 30 m*M* 3-(N-morpholino)propanesulfonic acid-potassium hydroxide (MOPS-KOH) (pH 7.8), 2 m*M* ethylenediaminetetraacetic acid (EDTA) and 2 m*M* ascorbate. The solution was filtered through four layers of miracloth (Calbiochem, Billerica, MA) and was then centrifuged for 5 min at 1000 *g*. The pellets were resuspended in the same buffer and washed twice by centrifugation under the same conditions. The subsequent steps for thylakoid subfractions were those of Kieselbach et al. (1998).

Plant growth conditions and treatments

Arabidopsis thaliana Columbia-0 ecotype (Col-0) was used in this study. Transgenic seeds were sterilized and planted in plates containing Murashige and Skoog (MS) medium with 1% sucrose. After stratification in the dark at 4°C for 2 days, the plates were transferred to a growth chamber under 16 h light/8 h dark cycles. For the soil-grown plants, 10-day-old seedlings grown on MS medium were transferred to soil and grown in the same growth chamber. For the HL conditions, 2-week-old seedlings were subjected to a light intensity of 880 µmol photons m^{-2} s^{-1} for 12 and 48 h. We used a light intensity of 100 µmol photons m^{-2} s^{-1} for 'normal light' (NL) for seedlings of the same age. Total RNA and chloroplast thylakoid membranes were isolated for reverse transcription-polymerase chain reaction (RT-PCR) and western analysis. The AtFKBP16-1 protein level response to HL stress was examined using 5-day-old seedlings of VC1, OE1 and OE2 grown under NL then transferred to HL for 10 days on MS medium. For the drought stress, 3-week-old, soil-grown young plants were not watered for 12 days.

In order to test abiotic stress-response expression of *AtFKBP16-1*, 10-day-old seedlings were treated with 200 m*M* NaCl, 400 m*M* mannitol, 10 µ*M* MV, 10 m*M* H_2O_2 and 100 µ*M* abscisic acid (ABA) for 0, 3, 6, 9, 12, 36, 48 and 72 h (NaCl and mannitol); for 0, 1, 3, 6, 9, 12, 36 and 48 h (MV and H_2O_2); or for 0, 1, 3, 6, 9, 12, 24 and 36 h (ABA). Total RNA isolation from each time point and first strand cDNA synthesis were performed as described in a previous study (Ahn et al. 2010). These cDNAs were used for templates in the 20 µl PCR reaction using AtFKBP16-1 gene-specific primers (5′-AAGAA GACTGTTGACGACGACGG-3′ and 5′-CTTCATTAACA GTCGGTGACAGGAC-3′). The expression of the Arabidopsis *actin* gene was used as an internal control using specific *Actin1* primers, 5′-AAGAAGACTGTTGACGAC GACGG-3′ and 5′-ATGGCGACTCTCTTCACT GCGA CGGTTCC-3′. Arabidopsis *RD29A* gene was used for expression of stress inducible marker gene by means of the primers, 5′-CTTCATTAACAGTCGGTGACAGGAC-3′ and 5′-AAGCTCCTTCTGCACCGGAACAACAG-3′.

Generation of AtFKBP16-1 transgenic plants

In order to generate the FKBP16-1 OE plants, the protein coding sequence of FKBP16-1 was C-terminally fused with a double hemagglutinin (HA) tag amplified by PCR using the primers, 5′-CCGGGATCC ATGGCGATGGCTATGGAGATTTCTCTTCCTTTCGTTG GATC-3′ and 5′-AACGTCGACTCAAGCGTAATCTGGA ACATGAACATCGTATGGGTAAGCGTAATCTGGAACA TCGTATGGGTATAATACTTTCAAGAGCTGG-3′. The FKBP16-1 2HA fusion product was cloned into the binary vector pCAMBIA1300 (Hajdukiewicz et al. 1994) under the control of a CaMV 35S promoter. Two independent homozygous lines of AtFKBP16-1 OE (OE1, OE2) and pCAMBIA vector controls (VC1, VC2) were identified in the T_3 generation. For the suppression of AtFKBP16-1, a 553 bp fragment (72–625 nt) of the open reading frame encoding inverted-repeat RNA (RNAi)

into dexamethasone (DEX) inducible pTA7002 vector (Aoyama and Chua 1997). The primers used were 5′-A ATACTCGAGTCAAAGACCAGAGGCTCGTTCGCATG-3′ and 5′-GGACGAATTCATGGCCTTAAGCGCGGGAA AATCAAG-3′ for antisense; 5′-ACTCGGATCC ATGGC CTTAAGCGCGGGAAAATCAAG-3′ and 5′-ACTCACTA GT TCAAAGACCAGAGGCTCGTTCGCATG-3′ for sense orientation. To confirm the gene silencing level in mature plants, isolated RNAs from 3-day leaves with or without DEX treatment were analyzed using RT-PCR with primers from another part of the FKBP16-1 sequence, 5′-AAGAAGACTGTTGACGACGACGG-3′ and 5′-CTTC ATTAACAGTCAGTGACAGGAC-3′. Two independent homozygous lines set of FKBP16-1 RNAi (R1, R2) and pTA7002 VCs (TA1, TA2) were identified in the T_3 generation.

Antibodies

The AtFKBP16-1 antibody was prepared from mature protein of FKBP16-1 (72–207 aa), was amplified and then expressed into the pGEX4T-1 vector (Novagen, Darmstadt, Germany). The FKBP16-1::GST fusion protein was expressed by 0.2 m*M* isopropylthio-β-galactosidase for 2 h, and then the recombinant proteins were purified with glutathione-sepharose 4B resin (GE Healthcare, Uppsala, Sweden) according to the manufacture protocol. Polyclonal antisera were raised from own FKBP16-1 soluble protein in rabbit. Photosystem antibodies, PsaL (AS06 108), PsaF (AS06 104) and PsaA (AS06 172) for PSI; PsbA (AS11 1786) and PsbD (AS06 146) for PSII; PC (AS06 141) and cytochrome *f* (AS08 306) for electron transfer; and Lhcb1 (AS01 004) for LHC detection were purchased from Agrisera (Vännäs, Sweden). The glutathione-S-transferase (GST) and HA antibodies were used from Santa Cruz Biotech (Santa Cruz, CA) and Sigma (St Louis, MO), respectively.

Thylakoid membrane preparation, BN-PAGE and two-dimensional PAGE

Chloroplast and thylakoid membranes were isolated as described in our previous work (Kim et al. 2012) and BN-PAGE was conducted as described by Fu et al. (2007). All steps of the sample preparation were carried out at 4°C. Thylakoid membranes were solubilized with mild detergent dodecyl maltoside (Sigma) for 10 min on ice to a final concentration of 1%, and the soluble fraction was transferred to a new tube after high speed centrifugation at 16 000 *g* for 30 min. The fractions were separated on 5–13.5% native PAGE, with sample buffer (0.05% Serva G, 3% sucrose, 100 m*M* Bis-Tris–HCl and 0.5 M 6-amino-caproic acid, pH 7.0) by electrophoresis at constant voltage (100 V, for 2 h) in a cold chamber. After BN-PAGE, a lane of the one-dimension (1-D) BN-PAGE gel was cut, and incubated for 20 min in 1× SDS-PAGE sample buffer for denaturation. The sliced 1-D gel was put on a 2-D, 12% SDS-PAGE for electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific, Waltham, MA). For immunoblot analysis, thylakoid proteins were loaded on equal chlorophyll basis (20 μg). Photosystem proteins were detected with Super Signal West Pico Chemiluminescence substrate (Thermo Scientific).

PsaL stability test by protease treatment

Solubilized thylakoid membranes (20 μg of chlorophyll) of VC and FKBP16-1 OE transgenic plants were incubated with thermolysin (2, 5 and 10 μg) on ice for 10 min in the presence of 25 m*M* Bis-Tris (pH 7.0), 20% glycerol and 1 m*M* $CaCl_2$. The reaction was terminated by adding EDTA to a final concentration of 50 m*M*. Proteins were denatured by SDS-PAGE sample buffer by boiling for 10 min, and were then separated on 12% SDS-PAGE. The photosystem proteins were detected by immunoblot using antibodies. For the effect of recombinant protein on protease accessibility, solubilized thylakoid membranes (20 μg of chlorophyll of wild-type Col-0) were incubated with recombinant GST or AtFKBP16-1 in proportion to concentrations of 0.25 μg (1×), 0.5 μg (2×) and 1.25 μg (5×) and then added 10 μg of thermolysin protease as in the previous assay for 10 min on ice. We used the same membrane for all the immunoblot by re-probing steps using 1 *M* NaOH.

PSII efficiency measurement

HL stress was assessed with 3-week-old plants under a light intensity of 880 μmol photons $m^{-2}\,s^{-1}$ using a Plant Efficiency Analyzer (Hansatech, Norfolk, UK) at 0, 1, 3 or 6 h. PSII efficiency (Fv/Fm) was analyzed as described by Kim et al. (2012) with six different detached leaves from the VC, and AtFKBP16-1 OE plants, at each time point. MV was applied to 3-week-old transgenic leaf discs from VC and to AtFKBP16-1 OE plants, with concentrations of 1, 2 and 5 μ*M* for 0, 0.5, 1 and 3 h to measure the maximum quantum yield of PSII. H_2O_2 was also applied to 3-week-old transgenic leaf discs from VC and AtFKBP16-1 OE plants with concentrations of 10 and 20 m*M* for 0, 0.5, 1 and 3 h.

Results

AtFKBP16-1 is, a ubiquitous chloroplast lumenal protein, involved in stress response

AtFKBP16-1, a chloroplast lumen FKBP-type IMM, contains a chloroplast-targeting transit peptide (1–71

aa), and a lumen-specific signal (RR), in its N-terminal region. It was initially detected during proteomic analysis of a chloroplast lumen fraction from Arabidopsis (Schubert et al. 2002, He et al. 2004). To assess targeting of AtFKBP16-1 into the chloroplast lumen, we separated isolated chloroplasts in thylakoid membrane and soluble lumen fractions and conducted immunoblot analysis of both materials using an AtFKBP16-1 antibody. For comparison, we confirmed the locations of PC and cytochrome *f*, a known lumenal soluble protein and thylakoid membrane protein, respectively. As expected, the AtFKBP16-1 protein localized to the thylakoid lumen fraction (Fig. 1A). The expression of *AtFKBP16-1* was detected in all tissues except roots, and was regulated by light induction (He et al. 2004). To determine the levels of AtFKBP16-1 during different physical locations and temporal stages, we analyzed immunoblots of AtFKBP16-1 during different growth stages, and its localization in various plant tissues. AtFKBP16-1 was maintained during all growth stages, from 1-week-old plantlets to 5-week-old, full-grown plants, although its level slightly decreased in mature plants with an age of 4 weeks or more (Fig. 1B). In addition, AtFKBP16-1 protein was in all green tissues, including leaves (young and old), stems, siliques and flowers, but was absent in roots (Fig. 1C). Because AtFKBP16-1 was located in chloroplasts and expressed only in aerial parts of the plant, the levels of AtFKBP16-1 protein were assessed to ensure that it was affected by light intensity and the duration of illumination. As shown in Fig. 1D, the protein level in plants illuminated for 48 h under HL (880 µmol photons m^{-2} s^{-1}) was much higher than in plants grown under NL (100 µmol photons m^{-2} s^{-1}) or under HL for only 12 h. Furthermore, in order to confirm that *AtFKBP16-1* is regulated by other abiotic stresses, we conducted RT-PCR analysis of *AtFKBP16-1* under osmotic-stress conditions, such as high NaCl and mannitol concentrations, and under oxidative stress caused by MV and H_2O_2. *AtFKBP16-1* transcript levels were increased in response to various abiotic stresses (see Fig. S1). These results suggest that AtFKBP16-1 is constitutively localized to the thylakoid lumen in the light and that this protein may play a role in photosynthesis under stress conditions.

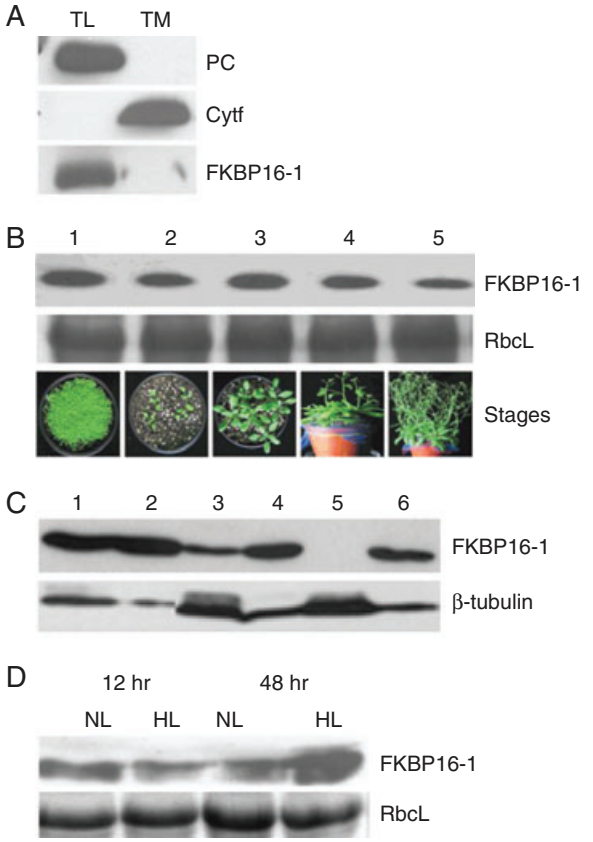

Fig. 1. AtFKBP16-1 is a ubiquitous chloroplast lumenal protein in green plant tissues. (A) Immunoblot analysis of AtFKBP16-1 in the thylakoid membrane and thylakoid lumen subfractions. TL, thylakoid lumenal fraction; TM, thylakoid membrane fraction; Cytf, cytochrome b_6f subunit. (B) Immunodetection of AtFKBP16-1 at various developmental stages. 1, 1-week-old seedlings; 2, 2-week-old young plants; 3, 3-week-old plants; 4, 4-week-old mature plants and 5, 5-week-old flowering plants. Rubisco large subunit (RbcL) protein was employed as a loading control. (C) Immunodetection of AtFKBP16-1 in various tissues. 1, 2-week-old leaves; 2, 4-week-old leaves; 3, flowers; 4, stems; 5, roots and 6, siliques. β-tubulin was used as a loading control. (D) Immunoblot analysis of AtFKBP16-1 under high light stress conditions. NL (100 µmol photons m^{-2} s^{-1}); HL (880 µmol photons m^{-2} s^{-1}). RbcL protein was employed as a loading control. Fifty micrograms of total protein were used in (B–D) for immunoblotting.

Increased AtFKBP16-1 levels cause tolerance against high light

As the expression of AtFKBP16-1 is induced by light (see Fig. S1) and the protein level is regulated by light intensity (Fig. 1D), we first checked HL stress tolerance. To obtain AtFKBP16-1 OE plants, the CaMV35S::*AtFKBP16-1* (tagged with 2HA) construct was introduced into wild-type Arabidopsis plants and the effect of this change was tested by western analysis using AtFKBP16-1 antibody. As shown in Fig. 2A, two OE transgenics (lines, OE1 and OE2) showed elevated levels of the protein relative to VC plants transformed with empty vector. The expression in VC (line, VC1) plant seemed absent, but this was because of the relatively strong expression of two OE lines (Fig. 2A). The endogenous AtFKBP16-1 protein level was detected only after extended exposure (see Fig. S3). To investigate whether OE transgenic lines show a response against the HL stress, we tested the effect on growth of 5-day-old seedlings, and quantified

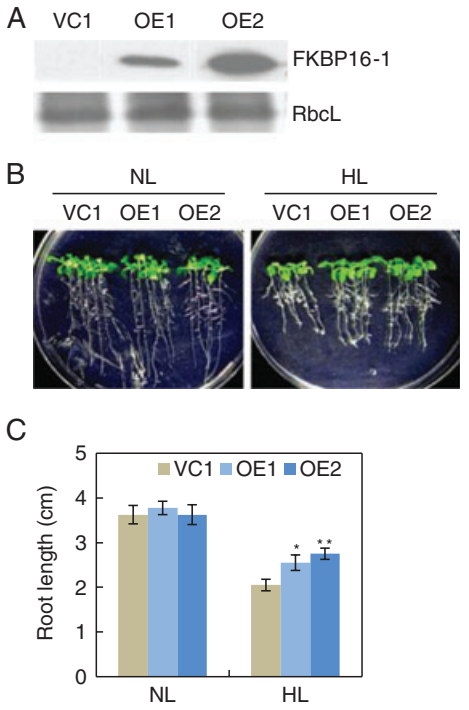

Fig. 2. Overexpression of AtFKBP16-1 enhanced tolerance to high light stress. (A) Immunodetection of overexpressed AtFKBP16-1 protein level. VC1, pCAMBIA vector control line; OE1 and OE, independent AtFKBP16-1 2HA-tagged homolines. (B) High light tolerant phenotype of AtFKBP16-1 OE plants. NL, 5-day-old transgenic seedlings were grown under normal light condition for 10 days; HL, 5-day-old seedlings were grown under high light condition for 10 days. (C) Root lengths of AtFKBP16-1-overexpressing, and vector control, seedlings under NL and HL. Five-day-old seedlings were grown under NL and HL. The root length of 7-day seedlings, grown under different light conditions, was measured. Each column contains an average of five replicates, and bars indicate standard deviations. Asterisks indicate significant differences from VC using *t*-test *P* value (**t*-test, with $P < 0.01$, ***t*-test, with $P < 0.001$).

the root length of VC (line, VC1) and OE (lines, OE1, OE2) seedlings in relation to HL stress (880 µmol photons m^{-2} s^{-1}). As we expected, the results showed that both OE transgenic lines grew better than the VC plants. The OE lines had shoots that were greener and bigger, and the roots that were longer than those of VC1 plants after HL treatments. In contrast, there was no meaningful difference in growth and root length between VC and OE lines under the NL condition (Fig. 2B, C). Furthermore, AtFKBP16-1 knock-down plants were more sensitive to HL stress (see Fig. S2). Taken together, these results indicated that AtFKBP16-1 is likely to play a role when chloroplast is placed in HL stressful conditions.

In addition, to determine the role that reactive oxygen species (ROS) plays in the AtFKBP16-1 mediated stress response, specifically with respect to photosynthesis, we measured the PSII efficiency (Fv/Fm values) of VC (line, VC1) and OE (lines, OE1 and OE2) AtFKBP16-1 transgenic plants under HL-, MV- and H_2O_2-stress conditions. Under HL stress, the two independent OE plants showed increased Fv/Fm values after 1–6 h, and especially at 6 h. The disparity in Fv/Fm values between the VC and OE plants increased with increasing treatment time, and the level of AtFKBP16-1 protein (see Figs. S3 and S4A). Treatment with MV also resulted in a higher Fv/Fm value in FKBP16-1 OE plants than in VC transgenic plants (see Fig. S4B). In addition, the FKBP16-1 OE plants showed more tolerance to another ROS stressor, H_2O_2, than did VC plants (see Fig. S4C). These data indicate that AtFKBP16-1 may play an important role in the photo oxidative defense pathway in plants under conditions of environmental stress.

AtFKBP16-1 affects the photosystem complex under HL stress

As increased expression of AtFKBP16-1 affects the acclimation of plants to HL stress (Fig. 2), we explored how AtFKBP16-1 functions within photosynthetic organs, especially under HL stress conditions. First, we compared the level of proteins within photosynthetic complexes, of VC (line, VC1) and AtFKBP16-1 OE (lines, OE1 and OE2) plants under HL stress, using BN-PAGE and 2-D analysis. As shown in Fig. 3A, the band intensities of PSI-LHCI-LHCII supercomplexes (I) and PSI-LHCI (II) were increased in OE1 and OE2 compared to the band intensities of the VC1 plants; even if the band intensities of PSII monomer (III) and CP43-less (IV) were not significantly different between VC and OE plants. By sequential 2-D analysis of 1-D gels, it was found that the OE2 plants showed a slight increase in levels of the subunits comprising PSI, whereas the amount of putative PsbB (see the Aro's paper for this reason; Rokka et al. 2005), a subunit of the PSII monomer, was not increased. Instead, it was slightly decreased in the OE2 line compared to the VC1 control plants (Fig. 3B, ◄). These results suggest that AtFKBP16-1 did not affect an early step in the generation of the photosynthetic complex unit, but rather affected plant acclimation by inducing a rapid transition of LHCII to the PSI-LHCI and PSI-LHCI-LHCII supercomplex under stress conditions (Fig. 3B).

Increased AtFKBP16-1 levels confer enhanced drought tolerance by stabilizing PsaL protein

Among the various abiotic stress tolerances of AtFKBP16-1 OE plants, drought tolerance was the most prominent. When watering of 3-week-old plants previously grown under normal conditions was stopped for 12 days, the VC

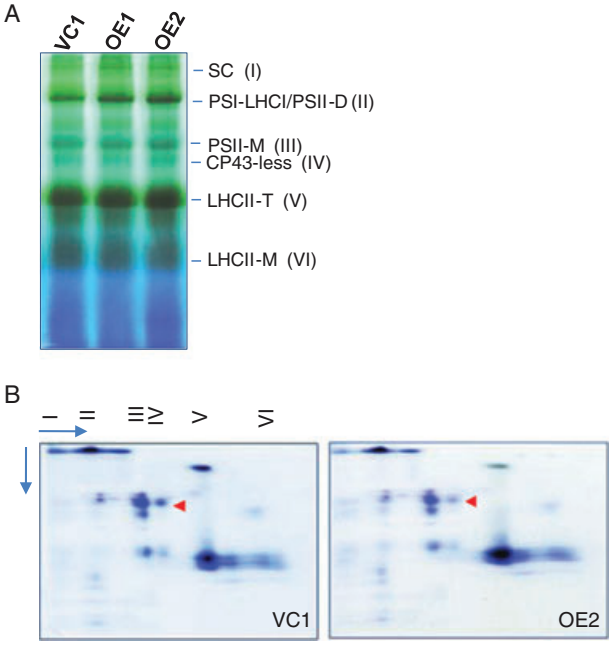

Fig. 3. Overexpressing AtFKBP16-1 affects PSI-LHCI and PSI-LHCI-LHCII photosystem complexes under high light conditions. (A) BN-PAGE analysis of AtFKBP16-1 OE plants under high light stress. Two-week-old seedlings were exposed to high light (880 μmol photons m^{-2} s^{-1}) for 72 h. Thylakoid membrane (20 μg chlorophyll) was solubilized with 1% dodecyl-β-maltoside and separated by 5–13% native gel. VC1, pCAMBIA vector control plants; OE1 and OE2 are independent AtFKBP16-1over-expression plants. SC (I), PSI-LHCI-LHCII supercomplex; PSI-LHCI/PSII-D (II), PSI-LHCII complex; PSII-M (III), PSII monomer; CP43-less (IV), CP-43 less PSII monomer; LHCII-T (V), light harvest complex trimer; and LHCII-M (VI), light harvest complex monomer. (B) Subsequent 2-D analyses of A samples by Coomassie staining. ◄indicates a putative PsbB subunit of PSII.

plants (lines, VC1 and VC2) stopped growing, exhibited defective growth with chlorophyll degradation, wilted and finally died. In contrast, the FKBP16-1 OE plants (lines, OE1 and OE2) exhibited better growth, with delayed wilting, higher biomass, lower incidence of chlorosis and other signs of enhanced drought tolerance compared to the VC plants. In a recovery assay in which plants were watered after withholding water for 1 week, the VC plants did not survive, even after 5 days of watering, but the FKBP16-1 OE plants recovered from drought stress within 1 day of water resumption (Fig. 4A). As another confirmation of the effect of AtFKBP16-1 OE plant on drought tolerance, four independent OE lines (OE1, OE2, OE3, and OE4) were tested at germination. Seeds were sown on wetted soil, and then water was withheld for 20 days. The VC seedlings ultimately died but all four independent OE line seedlings survived under the drought conditions (see Fig. S5). It was previously reported that FKBP16-1 interacts with PsaL, a subunit of the PSI complex, in a yeast two-hybrid assay (Gollan et al. 2011). To substantiate our notion that the increased expression of AtFKBP16-1 affects PsaL protein levels, we performed SDS-PAGE immunodetection of photosynthetic proteins in VC (line, VC1) and AtFKBP16-1 OE (lines, OE1 and OE2) transgenic plants under normal and drought conditions. As shown in Fig. 4B, the level of PsaL protein in FKBP16-1 OE plants increased little compared to that in VC plants under normal conditions, but PsaL protein levels were substantially higher in the OE lines under drought conditions. Other photosystem-related proteins did not exhibit notable quantitative changes. Indeed, the levels of PsaL in these lines increased proportionally to the FKBP16-1 protein levels in the OE lines. The levels of the two proteins, FKBP16-1 and PsaL, also correlated with the enhanced drought tolerance of the OE lines. This parallel up-regulation suggests that AtFKBP16-1 plays a role in the acclimation of photosynthesis to drought by maintaining an appropriate level of AtPsaL protein.

FKBP16-1 functions in maintaining PsaL stability

To determine how the function of FKBP16-1 is related to PsaL stability, we conducted a protease digestion assay in VC and AtFKBP16-1 OE thylakoid membranes; using thermolysin (Sigma) as described by Meurer et al. (1998) with some modifications. Solubilized thylakoid membranes (prepared using the conditions described above) were incubated on ice with either 2 or 5 μg of thermolysin, along with 1 m*M* $CaCl_2$, for 30 min, and the reactions were then terminated using 50 m*M* EDTA. PsaL protein is more sensitive to proteolysis than either PsaF or Lhcb1, as determined in a study of cyanobacteria (Chitnis and Chitnis 1993). When 5 μg of thermolysin was employed, most of the PsaL protein was degraded in the VC plants, but in the AtFKBP16-1 OE thylakoids, the PsaL protein resisted degradation after treatment with either 2 or 5 μg of thermolysin (Fig. 5A). Both PsaF and Lhcb1 remained undegraded after 30 min of treatment with either concentration of thermolysin. We also performed a protease accessibility assay using recombinant AtFKBP16-1 protein. In this assay, solubilized thylakoid membranes of wild-type Col-0 Arabidopsis plants were incubated with either GST control protein or AtFKBP16-1 recombinant protein in proportion to the concentration (1×, 2× and 5×) for protease digestion as in Fig. 5A, except that 10 μg thermolysin was used. PsaF, a subunit of PSI, remained in the undegraded form in the presence of GST or AtFKBP16-1 recombinant protein under the conditions described in Fig. 5A, but the Lhcb1 protein was degraded by 10 μg of thermolysin, regardless

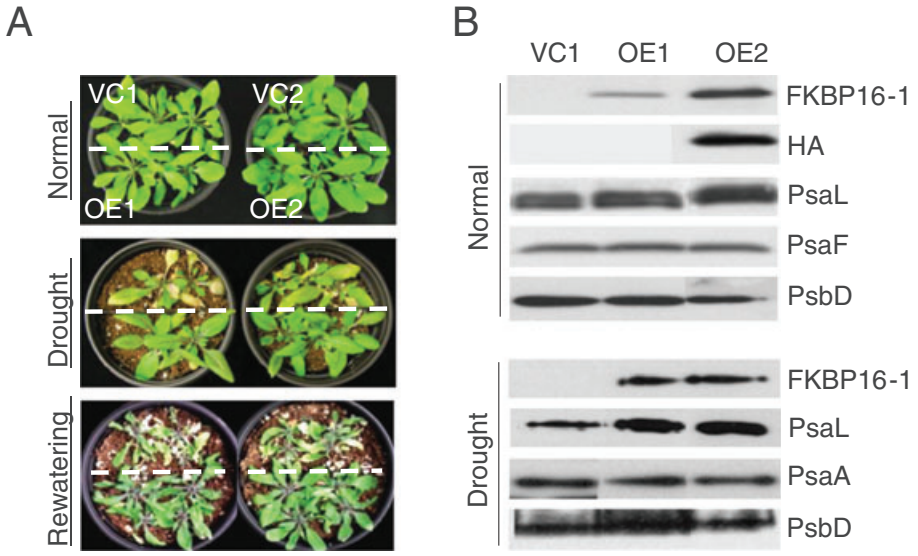

Fig. 4. AtFKBP16-1 overexpressing plants exhibit enhanced tolerance to drought stress by maintaining the stability of PsaL. (A) Drought tolerant phenotype of AtFKBP16-1 overexpression plants. VC1 and VC2, independent pCABMIA vector control plant lines; OE1 and OE2, 2HA-tagged independent AtFKBP16-1 OE plant lines. Normal, plants were grown under normal conditions; Drought, water was withheld from 3-week-old mature plants for 12 days; Rewatering, plants were watered after withholding water for 5 days. (B) Immunodetection of photosynthetic complexes under normal and drought stress conditions. Ten micrograms chlorophyll of thylakoid membranes were boiled in SDS sample buffer for 10 min for western analyses. PsaL, PsaA and PsaF antibodies were used to detect PSI subunits; PsbD antibody was used to detect PSII subunits and overexpressed 2HA-tagged FKBP16-1 in OE plants was detected with FKBP16-1 antiserum, and via its tag by an anti-HA antibody.

of the presence of GST or recombinant AtFKBP16-1 protein. The addition of recombinant AtFKBP16-1 greatly increased the stability of PsaL in the presence of thermolysin, and this enhancement was proportional to the amount of recombinant AtFKBP16-1 added (Fig. 5B). We cannot rule out the possibility that the exogenous recombinant protein was sensitive to protease digestion, although FKBP16-1 and GST proteins were detected on the same immunoblot, and the proportions of these proteins remained similar after protease digestion (data not shown). Therefore, the higher level of PsaL protein was not caused by the autoprotease activity of AtFKBP16-1. With these results, we demonstrate that AtFKBP16-1 functions to enhance PsaL protein stability in the thylakoid membrane under photosynthetic stress conditions.

PsaL protein level changes in response to abiotic stresses

Cyclic electron flow by supercomplex (PSI, LHCI, LHCII and Cyt b_6f) is crucial for a proper balance of ATP and NADPH in thylakoid stroma and for protecting their photosynthetic apparatus from photodamage (Iwai et al. 2010). PsaH, a subunit of PSI that functions as docking site between PSI and LHCII, also increased under drought stress (Zhang et al. 2009, our unpublished data). To assess the protein level of PsaL during abiotic stress, we conducted immunoblotting for PsaL during drought and MV stress conditions. As shown in Fig. 6A, PsaL protein levels increased in response to drought and MV stress, 6–24 h after treatment. A BN-PAGE/2-D analysis also showed higher levels of PsaL under drought conditions than under normal conditions. PsaL was mainly detected in a broad range of PSI-LHCI-LHCII supercomplexes and PSI-LHCI photosystems. In contrast, the level of PsbD protein, a subunit of the PSII complex, did not respond to drought stress (Fig. 6B). This result suggests that with PsaH, the PsaL protein may also play a role in the acclimation of plant photosystem under stress conditions probably by regulating the interaction of LHCII with PSI-LHCI.

Discussion

As mentioned above, it was previously thought that the movement of soluble proteins was very limited in the chloroplast lumenal space as a result of the distribution of PSI and PSII and the cytochrome b_6f photosystem complex. However, in recent years, proteomic analysis has revealed that there are more than 80 proteins in the chloroplast lumen, and that a great number of these soluble proteins are IMMs (Goulas et al. 2006, Peltier et al. 2006, Zybailov et al. 2008). Compared to

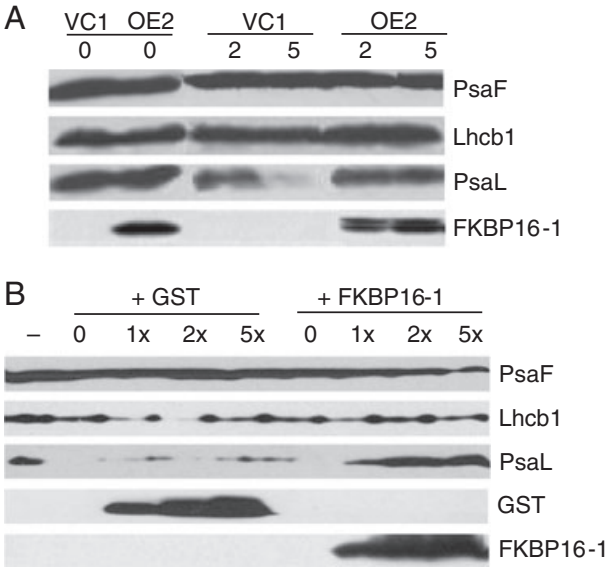

Fig. 5. AtFKBP16-1 regulates PsaL stability. (A) Analysis of PsaL stability in the presence of thermolysin in vector control and FKBP16-1 overexpression transgenic plants. VC1 and OE1, 2 are vector control and FKBP16-1 OE transgenic plant lines, respectively. 0, 2 and 5 were treated with 0, 2 and 5 μg thermolysin, respectively. PsaF and Lhcb1 were used for control proteins. FKBP16-1, 2HA-tagged AtFKBP16-1 protein. (B) Effect of recombinant FKBP16-1 on PsaL stability in a protease protection analysis. Thylakoid membranes from wild-type Col-0 were incubated with various concentrations of recombinant GST or FKBP16-1 for 10 min on ice and with 10 μg of thermolysin protease. 0, 1×, 2× and 5× were treated with 0, 0.25, 0.5 and 1.25 μg recombinant protein concentration, respectively. GST was used as a control recombinant protein.

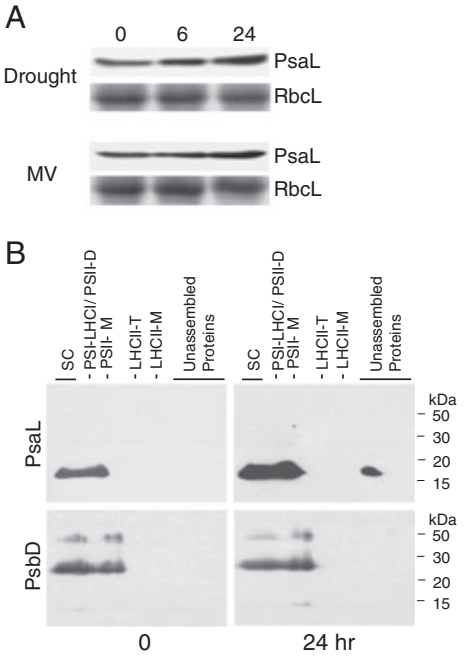

Fig. 6. Immunoblots of PsaL under drought and methyl viologen stress conditions. (A) Drought, water was withheld from 2-week-old wild-type Col-0 plants for 6 and 24 h; MV, 2-week-old wild-type Col-0 plants were treated with 10 μM MV for 6 and 24 h. RbcL protein was employed as a loading control. (B) BN-PAGE/2-D analysis of PsaL under normal and drought conditions. 0, normal condition; 24 h, drought for 24 h PsbD was employed as a loading control. SC, PSI-LHCI-LHCII supercomplex; PSI-LHCI/PSII-D, PSI-LHCII complex; PSII-M, PSII monomer; LHCII-T, light harvest complex trimer; LHCII-M, light harvest complex monomer.

the number of IMMs found in microorganisms or other organisms besides plants, the relatively high number of IMMs in green photosynthetic organisms is due to the presence of members of the IMM family in the photosynthetic apparatus. However, except for CYP38 and TLP40 (the CYP38 homolog in spinach), which are required for the assembly and stabilization of PSII, the precise physiological roles of the majority of the lumenal IMMs are not yet known. Furthermore, most of thylakoid lumenal IMMs do not possess five amino acids that are required for PPIase activity and a test for PPIase activity failed to reveal isomerization of synthetic peptides (Ahn et al. 2010, Gollan et al. 2011). Only two IMMs, FKBP13 and CYP20-2, contain all five of the amino acids required for PPIase activity, and these IMMs are responsible for almost all of the PPIase activity in the thylakoid lumen (Edvardsson et al. 2007, Ingelsson et al. 2009). Therefore, several questions remain about the function of a number of IMMs in the chloroplast; e.g.: Are the functions of these IMMs redundant? Is PPIase activity indispensable? Do these IMMs have functions other than PPIase activity?

Like most other thylakoid IMMs (Fig. 1), FKBP16-1s does not contain the conserved amino acids associated with PPIase activity (Ahn et al. 2010). Indeed, the IMM did not show PPIase activity when synthetic oligomers were used as a substrate (Gollan et al. 2011). However, it is still possible that FKBP16-1 has PPIase activity at a particular pH, e.g. at low pH under stress conditions such as HL, as the pH in the lumen can vary markedly from pH 4 to pH 7 (Chassin et al. 2002, Arnoux et al. 2009). In addition, in silico analysis of the promoter of *FKBP16-1* revealed the presence of *cis*-regulatory elements such as regulation of light (GATA box, GT-1 box), metabolite (TGACGT, TATCCAY, pyrimidine box, amylase box) and hormones (ARR1 element, CARE, CPB, LTRE, W-box and others) (Gollan and Bhave 2010). Actually, the expression of *AtFKBP16-1* increased in response to a variety of environmental stresses, including HL (see Fig. S1). Moreover, AtFKBP16-1 knock-down plants were more sensitive to HL stress, and AtFKBP16-1 OE plants were well-adapted to HL conditions (Figs. 2 and S2).

Previously, Gollan et al. (2011) used yeast two-hybrid analysis to show that FKBP16-1 interacts with the PsaL

subunit of PSI, particularly its C-terminal fragments (located on the luminal side of PsaL). The C-terminal region of PsaL may affect the interaction between PSI and LHCII, as revealed by a sequence comparison and the structural mapping of PsaL, which showed that the C-terminal fragment differed between plants (LPYFV[K]K) and cyanobacteria (VVDGIMTGLFN) (Vanselow et al. 2009). The conservation of the C-terminal fragment was confirmed through the analysis of additional plant PsaL amino acid sequences. All of the plant PsaL proteins possess a highly conserved amino acid sequence containing a proline residue in the C-terminal region. As previously mentioned, PPIase activity serves to isomerize the *cis* or *trans* form of proline residues during protein folding. Recently, however, some IMMs were found to regulate physiological activity through the isomerization of specific prolines in interacting proteins (Wang et al. 2010, Sarkar et al. 2011). Other questions remain, for instance: Is the proline present in the C-terminal fragment of PsaL essential for the interaction of this protein with FKBP16-1? Does *cis*/*trans* isomerization occur with this proline residue? If this isomerization indeed occurs, how does the interaction between FKBP16-1 and PsaL affects the PSI-LHCII interaction? While this study did not answer these questions, it showed that an increase in the FKBP16-1 level affected the level of PsaL, and that the strength of the interaction between FKBP16-1 and PsaL is proportional to the sensitivity of a plant to environmental stress (Fig. 4A, B). Also, FKBP16-1 was found to exist in a soluble form in the lumen, but to move to the PSI-LHCI or PSI-LHCI-LHCII supercomplex region under HL conditions (Cho et al., unpublished data), indicating that the interaction between FKBP16-1 and PsaL may only occur under stress conditions in the lumen. Finally, the interaction between FKBP16-1 and PsaL affected the PsaL level by inhibiting the degradation of PsaL by lumenal proteases (Fig. 5A, B). This finding may represent a novel function of IMMs; i.e. IMMs are involved in physiological regulation by affecting the stability of the proteins with which they interact.

In the photosynthetic organisms, Chlamydomonas and Arabidopsis, cyclic electron transport has been known to be crucial for proper balance of NADPH and ATP, and for protecting their photosynthetic apparatus from photo damage (Kramer et al. 2004, Shikanai 2007). Specifically, redistributing excitation energy through the migration of LHCII from PSII to PSI is called state transition, and is important for photo-acclimation. Phosphorylation and dephosphorylation in the LHCII N-terminal region were essential for the shuttle movement of LHCII between PSII and PSI (Depège et al. 2003). PsaH, a member of the subunits (PsaO, PsaL and PsaH) of PSI for the docking site of LHCII, was essential for state transitions to PSII regardless of LHCII phosphorylation (Lunde et al. 2000). We report here additionally that FKBP16-1, PsaL and PsaH levels were increased under stress and that their enhanced levels affected a transition of LHCII to the PSI-LHCI complex (Figs. 3 and 6). These results collectively suggest that FKBP16-1 plays an active role in maintaining PsaL stability under stress, and the possibility of the presence of another 'fine tuning mechanism' including FKBP16-1, PsaL and PsaH in regulation of LHCII state transitions, besides the known state transition by phosphorylation.

Acknowledgements – This work was supported by The Next Generation of Bio Green 21 Project, The National Center for GM Crops (PJ009043) from RDA, and KRIBB Initiative Program to H. S. C. and was also supported by The National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2100-0013767) to J. C. A.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. AtFKBP16-1 transcriptional levels under various abiotic stress conditions.

Fig. S2. Suppression of AtFKBP16-1 level caused high light sensitivity.

Fig. S3. AtFKBP16-1 overexpression levels in transgenic plants.

Fig. S4. Overexpressing AtFKBP16-1 plants showed enhanced tolerance to oxidative stresses.

Fig. S5. Overexpressing AtFKBP16-1 plants confer enhanced drought tolerance.

Edited by A. Krieger-Liszkay